

# Tobacco Nectaries Express a Novel NADPH Oxidase Implicated in the Defense of Floral Reproductive Tissues against Microorganisms<sup>1[OA]</sup>

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Hydrogen peroxide produced from the nectar redox cycle was shown to be a major factor contributing to inhibition of most microbial growth in floral nectar; however, this obstacle can be overcome by the floral pathogen *Erwinia amylovora*. To identify the source of superoxide that leads to hydrogen peroxide accumulation in nectary tissues, nectaries were stained with nitroblue tetrazolium. Superoxide production was localized near nectary pores and inhibited by diphenylene iodonium but not by cyanide or azide, suggesting that NAD(P)H oxidase is the source of superoxide. Native PAGE assays demonstrated that NADPH (not NADH) was capable of driving the production of superoxide, diphenyleneiodonium chloride was an efficient inhibitor of this activity, but cyanide and azide did not inhibit. These results confirm that the production of superoxide was due to an NADPH oxidase. The nectary enzyme complex was distinct by migration on gels from the leaf enzyme complex. Temporal expression patterns demonstrated that the superoxide production (NADPH oxidase activity) was coordinated with nectar secretion, the expression of Nectarin I (a superoxide dismutase in nectar), and the expression of *NOX1*, a putative gene for a nectary NADPH oxidase that was cloned from nectaries and identified as an *rbohD*-like NADPH oxidase. Further, in situ hybridization studies indicated that the NADPH oxidase was expressed in the early stages of flower development although superoxide was generated at later stages (after Stage 10), implicating posttranslational regulation of the NADPH oxidase in the nectary.

Many plants produce floral nectar, a rich source of carbohydrates, amino acids, and other metabolic components, as a reward for pollinator visitation. Because flower visitors are not sterile (Evans and Armstrong, 2006), they not only can transfer pollen during these visits but also transfer any microorganisms that they carry (Ferrari et al., 2006; Shafir et al., 2006). Nectar is a metabolically rich biological fluid containing sugars, vitamins, amino acids, organic acids, and metal ions

(Baker and Baker, 1971; Heinrich, 1989), all of which are utilized by microbes for growth. Despite the facts that flowers remain open, offering metabolically rich fluids over an extended timeframe, and that microbes are vectored into the plant's reproductive tract, infections of the gynoecium are relatively rare in plants. This implies that there must be an active defense system in nectar to reduce such infections. To examine this in greater detail, we began studies to determine whether there were proteins that accumulated in the nectar of plants. Over the past decade, we have identified a group of at least five proteins that accumulate in the nectar of ornamental tobacco (*Nicotiana langsdorffii* × *Nicotiana glauca*) plants (Carter et al., 1999; Carter and Thornburg, 2000, 2004b, 2004c; Naqvi et al., 2005). Together these proteins function in the nectar redox cycle, a novel biochemical pathway that produces very high levels of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>; Carter and Thornburg, 2004a) and permits the plant to deal with the resulting high concentrations of H<sub>2</sub>O<sub>2</sub>.

These proteins, termed Nectarins, accumulate in nectar to concentrations of 250 µg/mL of nectar. Two of these proteins, Nectarin I and Nectarin V, actively generate H<sub>2</sub>O<sub>2</sub> via different mechanisms. Nectarin I is a germin-like protein that has manganese superoxide dismutase activity (Carter and Thornburg, 2000; Lou and Baldwin, 2006). It disproportionates superoxide into H<sub>2</sub>O<sub>2</sub> and oxygen. Nectarin V is a berberine bridge

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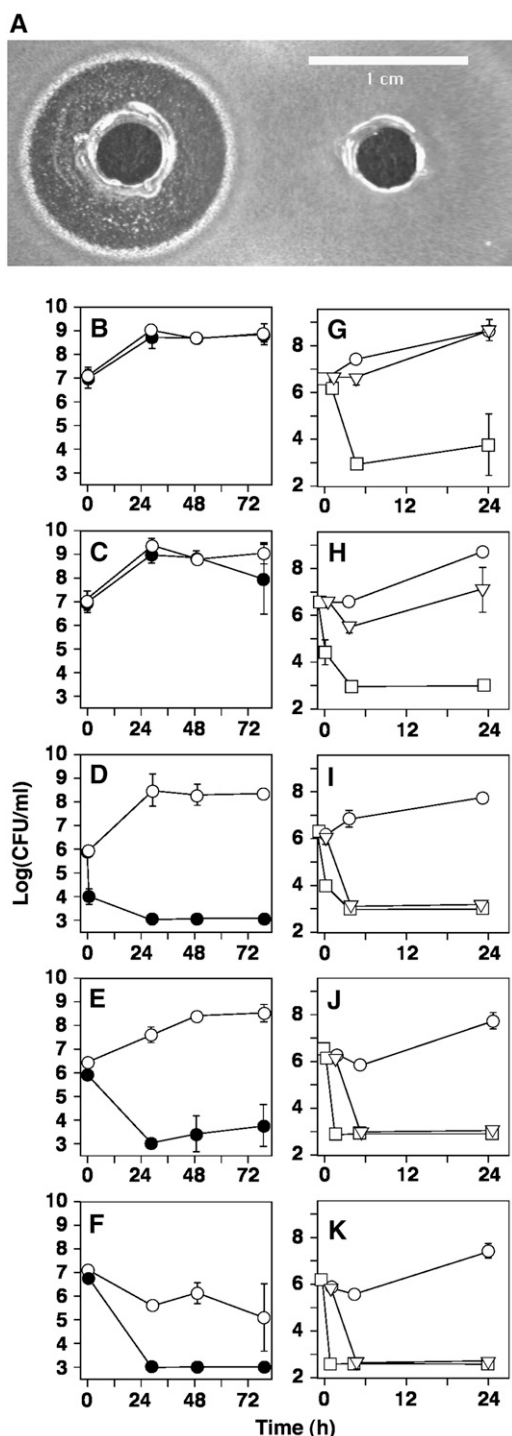
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**Figure 1.** Effect of nectar  $\text{H}_2\text{O}_2$  on the growth of microorganisms. A, Left, A lawn of *oxyR<sup>-</sup>* *E. coli* was plated and 10  $\mu\text{L}$  of nectar was added to the central well. After overnight development, a ring of growth inhibition was observed (left) or the nectar was incubated for 15 min with 1  $\mu\text{L}$  of 1 mg/mL catalase to destroy the  $\text{H}_2\text{O}_2$  prior to placement in the well (right). B to F, Growth of selected bacterial strains in raw nectar (black circles) or nectar that was preincubated (15 min) with 1  $\mu\text{L}$  of 0.5 mg/mL catalase (white circles). Each point represents the mean  $\pm$  SD ( $n = 4$ ). Data are representative of two independent experiments. G to K, Survival of selected bacterial strains in the absence (circles) or in the presence of 4 mM (triangles) and 40 mM (squares)  $\text{H}_2\text{O}_2$  in a minimal

enzyme-like protein that has Glc oxidase activity. It efficiently produces  $\text{H}_2\text{O}_2$  at the expense of nectar Glc. The net result of these two enzymes functioning in the nectar of ornamental tobacco is that  $\text{H}_2\text{O}_2$  accumulates to very high levels, up to 4 mM (Carter and Thornburg, 2000). This level is 40 times the level produced by human neutrophils in response to microbial attack (Prince and Gunson, 1987). We have previously proposed (Thornburg et al., 2003; Carter and Thornburg, 2004a) that the production of  $\text{H}_2\text{O}_2$  by the nectary serves to maintain floral nectar in a microbe-free state to protect the gynoecium, ovules, and developing seeds from pollinator and airborne pathogens. This study was initiated to evaluate the protective role of  $\text{H}_2\text{O}_2$  in plant nectars and to identify the source of superoxide that feeds into the nectar redox cycle.

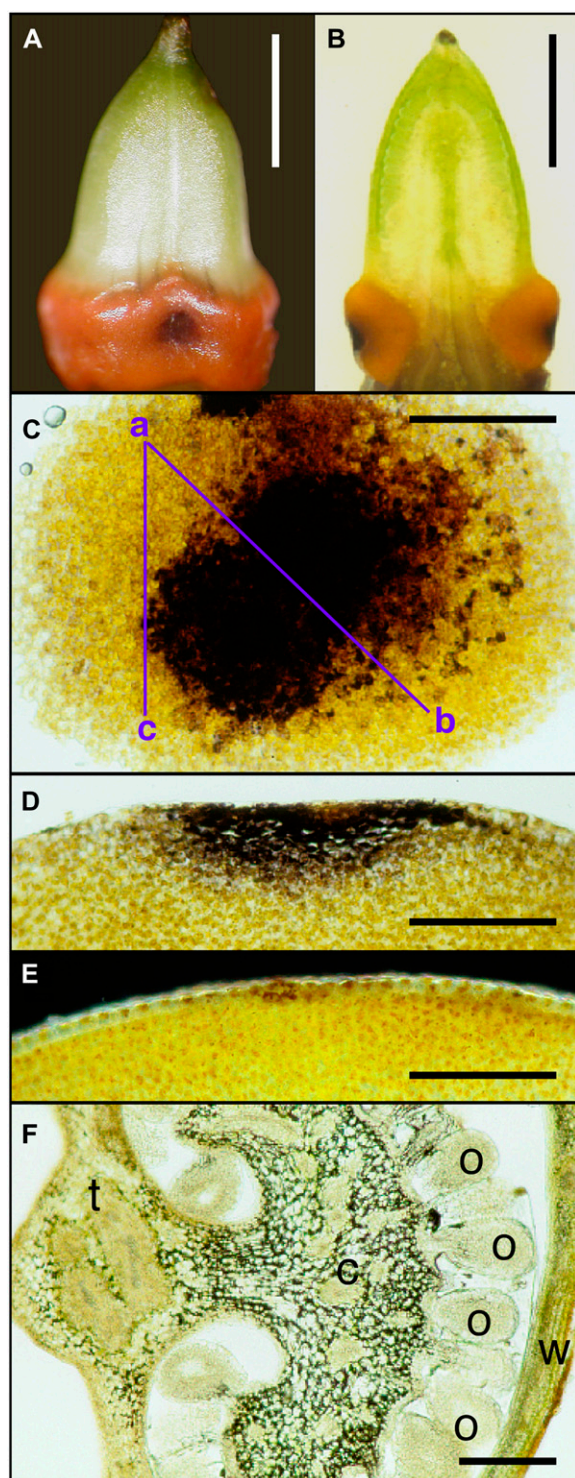
## RESULTS

### $\text{H}_2\text{O}_2$ in Ornamental Tobacco Nectar Is Toxic to Microorganisms

We have previously determined that the nectar of ornamental tobacco plants contains  $\text{H}_2\text{O}_2$  at 4 mM. To determine whether this level of  $\text{H}_2\text{O}_2$  had a protective function in nectar, we examined nectar alone to see if it could inhibit bacterial growth. As shown in Figure 1A, the left-hand zone shows a lawn of *Escherichia coli* surrounding a well into which was placed 10  $\mu\text{L}$  of raw nectar. After overnight incubation, there was a dramatic zone of clearance, demonstrating that tobacco nectar does indeed inhibit bacterial growth. To determine whether this growth inhibition was due to the presence of  $\text{H}_2\text{O}_2$  in the nectar, we conducted a short preincubation of nectar with catalase and repeated the experiment. This treatment eliminates this zone of inhibition by catalase and demonstrates that  $\text{H}_2\text{O}_2$  is responsible for inhibiting *E. coli* growth (Fig. 1A, right).

We also evaluated the potential inhibitory activity of nectar toward microorganisms commonly associated with plants. The selected organisms included a major plant pathogen that invades plants through the flowers, *Erwinia amylovora*; two plant-associated saprophytic species that are known antagonists of *E. amylovora*, *Pantoea agglomerans* (previously *Erwinia herbicola*) and *Pseudomonas fluorescens* (Pusey and Curry, 2004); a foliar pathogen of beans, *Pseudomonas syringae* pv *syringae*; and an animal pathogen that has been shown to be capable of colonizing plant leaves, *Salmonella typhimurium* (Brandl and Mandrell, 2002). As can be seen in Figure 1, B and C, the growth of the *E. amylovora* and *P. agglomerans* strains was not inhibited

medium. Each point represents the mean  $\pm$  SD ( $n = 3$ ). Data are representative of two independent experiments. The limit of detection was 3 log (colony forming units/mL). B and G, *E. amylovora*; C and H, *P. agglomerans*; D and I, *P. syringae*; E and J, *P. fluorescens*; and F and K, *S. typhimurium*.



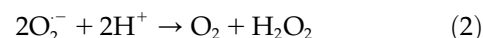
**Figure 2.** Staining of superoxide in mature Stage 12 floral nectaries of ornamental tobacco with NBT. A, Side view of gynoecium with orange basal nectary stained with NBT. B, View of a median-longitudinal slice through gynoecium and basal nectary taken after rotating the gynoecium in A by 90 degrees, showing the symmetrical staining pattern associated with both nectary pore regions. C, Tangential vibratome section of nectary pore region. a-b and a-c indicate two transects representing vibratome planes of sections shown in D and E, respectively. D, Cross section through the a-b transect of the NBT-stained

by the ornamental tobacco nectar, whereas the growth of *P. syringae*, *P. fluorescens*, and *S. typhimurium* strains was inhibited (Fig. 1, D–F). When the nectar was pretreated with catalase, however, the growth of *P. syringae*, *P. fluorescens*, and *S. typhimurium* in the catalase-treated nectar was significantly improved (Fig. 1, D–F), indicating that  $H_2O_2$  was a major toxic agent in the nectar.

We evaluated the inhibitory effect of  $H_2O_2$  on these microorganisms by monitoring culturable cell numbers over time following exposure to  $H_2O_2$ . Figure 1, G and H, shows that *E. amylovora* and *P. agglomerans* both tolerated exposure to 4 mM  $H_2O_2$ , consistent with their growth in untreated nectar, although they were killed by 40 mM  $H_2O_2$ . In contrast, *P. syringae*, *P. fluorescens*, and *S. typhimurium* were all rapidly killed by 4 mM  $H_2O_2$ , and more rapidly by 40 mM  $H_2O_2$  (Fig. 1, I–K).

#### Localization of Superoxide Production to the Nectary Pores in Ornamental Tobacco Nectar

The production of  $H_2O_2$  for the nectar redox cycle has been proposed to be a two-step process in which superoxide is produced from molecular oxygen (Eq. 1) and, subsequently, the superoxide is disproportionated into  $H_2O_2$  and molecular oxygen (Eq. 2).



To evaluate the source of superoxide, we investigated where superoxide was produced in nectaries. These studies relied on staining for superoxide using nitroblue tetrazolium (NBT). When reduced, NBT forms a purple-brown precipitate (blue-formazan) that localizes the site of superoxide production (Maly et al., 1989; Frahry and Schopfer, 2001; Kanehira et al., 2003). When mature, whole nectaries (Stage 12) were stained for superoxide (Fig. 2A); the superoxide-generating activity was localized to a small region on each lateral face of the nectary. These intensely staining regions mark the locations of the two nectary pores. Each nectary pore is a region of the nectary that is characterized by the presence of large numbers of stomata (O'Brien et al., 1996; Gaffal et al., 1998; Thornburg et al., 2003). Staining, indicative of superoxide, is present at each pore (Fig. 2B). Each of these staining regions is localized in the epidermal and subepidermal tissues of the nectary at the point overlying the junction of carpel fusion. In mature flowers,

nectary pore region showing depth of staining, flattened outer appearance of pore, and flattened, stained cells. E, Cross section a–c transect showing the periphery of the pore region with robust, unstained cells. F, Portion of cross section through gynoecium at the level of the locules showing the placenta or transmission tract with vascular bundles (t), ovary wall (w), and ovules (o). Some of the connective tissue (c) also stains positive for NBT. Bars = 1 mm on A and B; 200  $\mu$ m on C, D, and E; and 250  $\mu$ m on F.



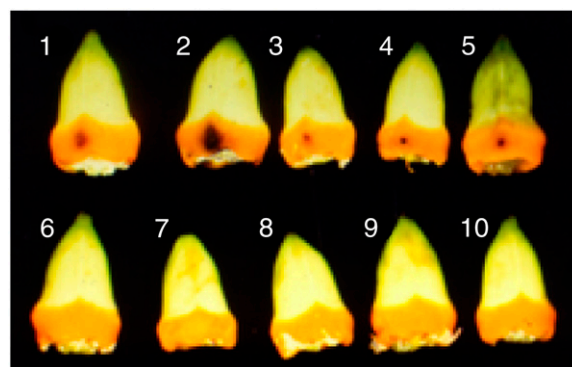
the stomata in these two zones are constitutively open (Thornburg et al., 2003) and are not responsive to stimuli (such as abscisic acid or  $H_2O_2$ ) that regulate leaf stomata (C.J. Carter and R.W. Thornburg, unpublished data). There are no stomata present in the nectary epidermis beyond these superoxide-staining regions, nor are there any stomata present in the ovary epidermis. To examine the staining in greater detail, we cut thick paradermal sections through the nectary for light microscopy. NBT staining is localized to the regions subtending the stomata of the nectary pores and is more intense toward the center of these regions. To better evaluate the expression of superoxide in the regions underlying the nectary pores, we cut medial sections along the two transects (a–b and a–c) as shown in Figure 2C. The (a–b) transect (Fig. 2D) is made through the center of the nectary pore and shows that the accumulation of superoxide is localized to the underlying region. The bowl-shaped staining pattern demonstrates that the superoxide production is localized to the nectary pore regions. The (a–c) transect (Fig. 2E) is at the edge of the stained area and confirms that superoxide production is localized to the nectary pore region proper and does not extend throughout the nectary.

One concern in interpreting this experiment is the possibility that the NBT substrate might not penetrate the tissue to react with superoxide. However, the NBT did successfully penetrate the ovary to stain the vascular tissues several cell layers below the epidermis in the ovary (Fig. 2F). Thus, we concluded that the NBT is penetrating the tissues and is accurately identifying the source of superoxide in the nectary.

#### Enzymatic Source of Superoxide Generation in Nectaries

After confirming the production of superoxide in the nectary pore region, we sought to determine the enzymatic source of superoxide production. Many plants utilize either a NAD(P)H oxidase (Lamb and Dixon, 1997; Keller et al., 1998) or a NAD(P)H peroxidase (Vianello and Macri, 1991; Bolwell et al., 1998) to produce superoxide. Both of these enzymes are inhibited by diphenyleneiodonium chloride (DPI). To evaluate whether DPI could inhibit the superoxide production from the nectary tissues, nectaries were stained for superoxide with NBT as before (Fig. 3, 1–5), and a second set of nectaries was preincubated with 50  $\mu$ M DPI for 10 min prior to staining with NBT (Fig. 3, 6–10). As shown, treatment with DPI significantly reduced superoxide production in the nectary pore region, suggesting that either an NAD(P)H oxidase or an NAD(P)H peroxidase may be responsible for superoxide production.

Based upon these results, we next examined superoxide production in nectary tissue extracts. Extracts were subjected to native PAGE and subsequently stained for superoxide production. In the absence of the electron donor or with added NADH, there was little superoxide produced (Fig. 4A, lanes 1 and 2). In

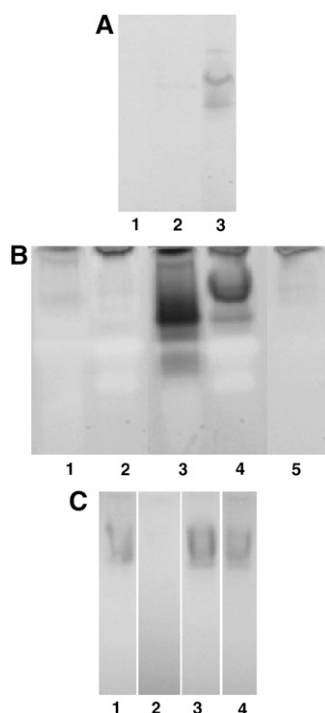


**Figure 3.** Inhibition of superoxide production by DPI. Top row, 1 through 5, mature Stage 12 gynoecia stained with NBT. Bottom row, 6 through 10, mature Stage 12 gynoecia stained with NBT, as in the top row, except the solution also contained 50  $\mu$ M DPI.

contrast, in the presence of NADPH, at least two different superoxide-producing bands were identified (Fig. 4A, lane 3). Other nucleotide cofactors ( $FADH_2$ ,  $NAD^+$ , and  $NADP^+$ ) did not elicit superoxide production (data not shown).

To determine whether the nectary NADPH oxidase activity is similar to that present in leaves, both leaf and nectary tissue extracts were examined following native PAGE separation. In Figure 4B, lanes 1 and 2 show leaf and nectary tissues without added NADPH, and lanes 3 and 4 show leaf and nectary tissues in the presence of NADPH. There are two bands clearly discernable in lane 4. The lower of these bands comigrates with the major superoxide-generating activity found in leaves. However, the major nectary superoxide-producing activity migrates at a different position in the gel. Because this assay is based on the migration of enzyme complexes through native gels, we can conclude that the enzymes producing activity in leaves and in nectaries are different and may contain different or additional polypeptide subunits that are responsible for this shift in enzymatic activity. When the nectary superoxide-producing complexes were tested for sensitivity to DPI, we observed complete inhibition of superoxide production (Fig. 4B, lane 5).

To distinguish between NADPH oxidase and NADPH peroxidase, both of which are sensitive to DPI (Vianello and Macri, 1991; Lamb and Dixon, 1997; Bolwell et al., 1998; Keller et al., 1998), we examined the sensitivity of this activity to sodium cyanide and sodium azide. These compounds inhibit NADPH peroxidase but not NADPH oxidase (Bolwell et al., 1995, 1998; Frahy and Schopfer, 1998). As shown in Figure 4, the superoxide generation activity of the nectary tissues was not inhibited by either 10 mM potassium cyanide (Fig. 4C, lane 3) or 10 mM azide (Fig. 4C, lane 4); however, it was inhibited by 6  $\mu$ M DPI (Fig. 4C, lane 2). These studies imply that superoxide is enzymatically produced in nectaries by an NADPH oxidase enzyme complex that is qualitatively different from the NADPH oxidase found in foliage.



**Figure 4.** Native PAGE assay for NADPH oxidase. A, NADPH dependency of superoxide production. Approximately 12 nectaries were homogenized in 550 mL of 1 M NaCl, 50 mM sodium phosphate, pH 6.8, and centrifuged at 10,000g for 15 min at 4°C. Protein (100 µg) from each supernatant was electrophoresed on native, nondenaturing polyacrylamide gels. The gels were then stained with 0.5 mg/mL NBT for 30 min at 20°C. Nectary tissues were amended with: lane 1, no added dinucleotides; lane 2, 140 mM NADH; and lane 3, 140 mM NADPH. B, Comparison of enzyme activities in leaf and nectaries. Lanes 1 and 3 contained 100 µg of leaf proteins, and lanes 2, 4, and 5 contained 100 µg of nectary proteins that were amended with: lanes 1 and 2, no added nucleotides; lanes 3 and 4, 140 mM NADPH; and lane 5, 140 mM NADPH plus 6 mM DPI. C, Inhibition of NADPH oxidase activity. All lanes contained 100 µg of extracted nectary proteins that were incubated with NBT containing 140 mM NADPH and were preincubated with: lane 1, no inhibitor; lane 2, 10 mM DPI for 10 min; lane 3, 10 mM NaCN for 15 min; and lane 4, 10 mM NaN<sub>3</sub> for 15 min.

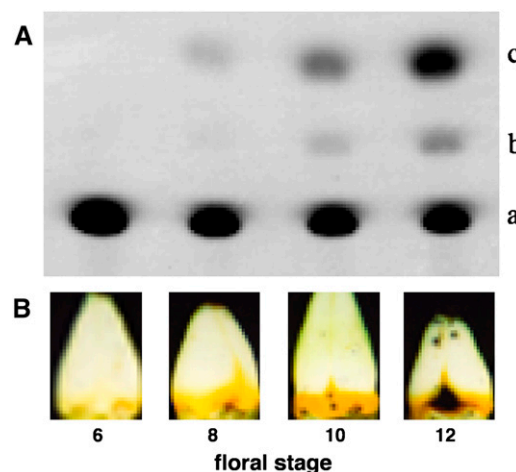
#### Temporal Expression of Superoxide-Generating Activity in Floral Development

To evaluate the temporal pattern of expression of the NADPH oxidase in the nectary, we stained nectaries from different developmental stages for superoxide. In tobacco, flower development has been divided into 12 different stages (Koltunow et al., 1990). Stage 1 is a tight bud and Stage 12 represents a mature open flower. Within this developmental timeframe, nectar begins to be secreted at about Stage 10, approximately 12 to 18 h prior to anthesis (Stage 12). As a marker of nectary development, we also evaluated when Nectarin I was expressed. Nectarin I is the major soluble nectar protein and catalyzes the second step (superoxide dismutase) in the production of H<sub>2</sub>O<sub>2</sub> (Eq. 2). Figure 5A shows the expression of a *NECI*-CAT con-

struct in transgenic tobacco plants at various stages of floral development (Carter and Thornburg, 2003). To assay the expression of the CAT protein, acetylated forms of radiolabeled chloramphenicol were monitored by thin-layer chromatography following incubation of free chloramphenicol with acetyl-CoA and 100 µg of total protein extracted from nectaries at each stage of development (Carter and Thornburg, 2003). As shown, expression of Nectarin I begins at Stage 8, some 24 to 36 h prior to anthesis and about 12 to 18 h prior to the beginning of nectar secretion. Thus, the pattern of expression driven by the *NECI* promoter shows that this gene is expressed slightly prior to the advent of nectar secretion. In the lower section (Fig. 5B), we see gynoecia dissected from flowers at these same developmental stages and stained for superoxide production with NBT. We see very little expression of superoxide-generating activity prior to floral Stage 10. After that, there is a significant and dramatic increase in the production of superoxide by Stage 12. Based on these data, we conclude the timing of expression of *NECI* (superoxide dismutase) precedes the advent of nectar secretion, and is temporally coordinated with the production of superoxide and thus of NADPH oxidase activity.

#### Cloning of a NADPH Oxidase Fragment

To better understand the role of NADPH oxidase in the superoxide generation process within the nectary, we attempted to isolate a cDNA encoding the nectary-expressed enzyme. To accomplish this, we aligned a



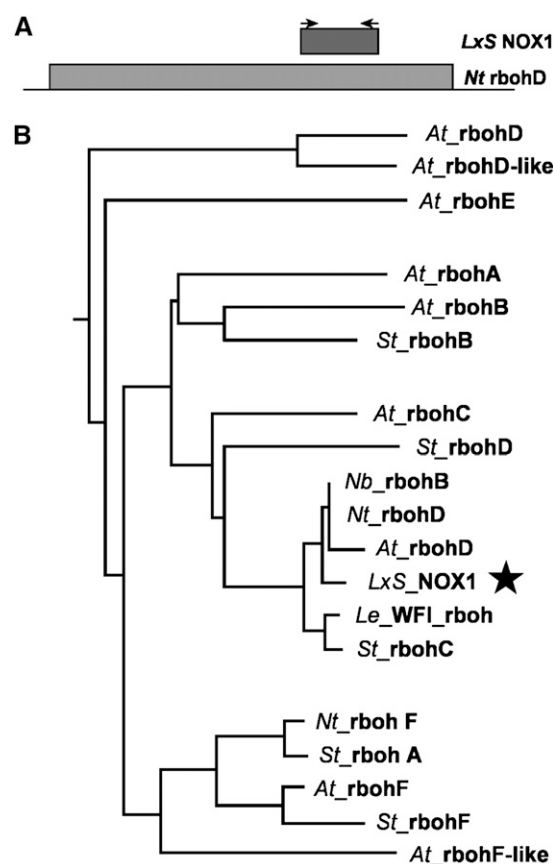
**Figure 5.** Coordination of Nectarin I expression with superoxide production. A, Expression of chloramphenicol acetyl transferase in different developmental stages of floral nectaries from Tr461 transgenic plants (*NECI*-CAT; Carter and Thornburg, 2003). The floral stages are Stage 6, mid-filling stage, presecretory; Stage 8, late-filling stage, presecretory; Stage 10, early maturation stage, just prior to nectar secretion; and Stage 12, fully secretory nectaries at anthesis. a, Non-acetylated [<sup>14</sup>C]chloramphenicol; b, [<sup>14</sup>C]chloramphenicol-L-acetate; and c, [<sup>14</sup>C]chloramphenicol-3-acetate. Gynoecia from different staged (6, 8, 10, and 12) flowers stained with NBT as in Figure 2.

series of NADPH oxidase sequences from various solanaceous species to identify regions that shared high identity. This strategy pinpointed several regions that were commonly conserved among these sequences. From these sequences we identified a series of eight degenerate oligonucleotides. These oligonucleotides were then used to amplify cDNAs from nectary-expressed mRNA using reverse transcription (RT)-PCR. mRNA isolated from Stage 12 nectaries (flowers at anthesis) was reverse transcribed, and the first-strand cDNA was used for RT-PCR using the degenerate oligonucleotides. The majority of the oligonucleotides failed to amplify a cDNA from the first-strand cDNA. However, one pair of oligonucleotides, nox1 and nox8 (5'-ACN-GGNTTYAAYGCNTTYTGGTA-3' and 5'-NGGNGT-NGCNCDDATNCC-3') permitted the successful amplification of a 601-nucleotide fragment. When the translated amino acid sequence from this fragment was aligned with the *Nicotiana tabacum* NADPH oxidase D (respiratory burst oxidase homolog D) sequence, this fragment corresponded to amino acids 545 to 754 of the 939-amino acid protein (Fig. 6A).

To evaluate the relationship of the nectary-expressed NADPH oxidase clone with other NADPH oxidases, we evaluated the phylogenetic relationship of these clones (Fig. 6B). The LxS NOX1 clone falls into a clade containing the Arabidopsis (*Arabidopsis thaliana*) gene At4g47910 (*rbohD*), several *Nicotiana* NADPH oxidase genes, and the tomato (*Lycopersicon esculentum*) white fly-inducible NADPH oxidase. This group of enzymes is expressed in a variety of tissues, including foliage, and some members of this group are induced in response to pathogen or insect attack (Simon-Plas et al., 2002; Yoshioka et al., 2003).

### Expression of the NADPH Oxidase

To evaluate the expression of the nectary NADPH oxidase, we used in situ hybridization to localize the expression of the NOX1 clone. However, to provide a useful comparison, we performed in situ hybridization using the Nectarin I (*NEC1*) clone. A 318-nucleotide fragment of *NEC1* was subcloned into a pGEM vector, pRT538, that provided both sense and antisense strands by transcription from the SP6 and T7 promoters, respectively. The transcribed antisense strand that should hybridize with the native mRNA failed to hybridize in immature Stage 6 nectaries (data not shown). However, mature nectaries intensely accumulated a purple-brown stain, indicating that the *NEC1* mRNA is actively expressed at Stage 12 (Fig. 7, A and B). Within the nectary there are two cell types: a single cell layer of epidermis and the remainder of the nectary, which is composed of special parenchyma cells. The *NEC1* mRNA accumulates in both of these cell types (Fig. 7B shows the special parenchyma). To confirm the specificity of the interaction, we also hybridized with the *NEC1* sense strand, produced from the SP6 promoter. No hybridization was observed with the sense strand in either the mature Stage

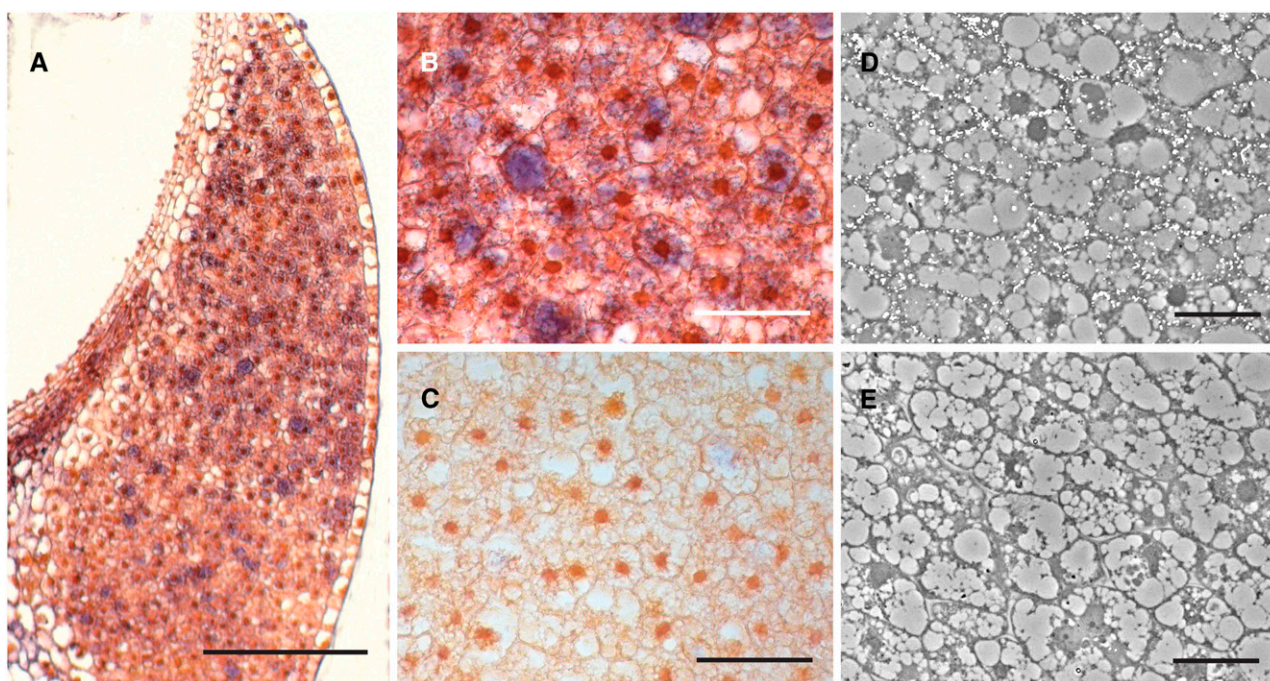


**Figure 6.** Nectary-expressed NADPH oxidase clone. A, Alignment of the cloned NOX1 clone with the *N. tabacum* *rbohD* clone (GenBank AF506374). B, Phylogenetic analysis of NOX1 and related *gp91phox* genes. This analysis was restricted to the homologous 200-amino acid region from each of these clones. The ClustalW analysis was conducted online at the Kyoto University Bioinformatics Center Web site (<http://clustalw.genome.jp/>). Clones used in this analysis (with GenBank or TAIR identifiers) were as follows: LxS NOX1, DQ497543; *At\_rbohA*, At5g07390; *At\_rbohB*, At1g09090; *At\_rbohC*, At5g51060; *At\_rbohD*, At3g45610; *At\_rbohD-like*, At5g60010; *At\_rbohE*, At1g19230; *At\_rbohF*, At1g64060; *At\_rbohF-like*, At4g11230; *Le\_WFI\_rboh*, AF148534; *Nb\_rbohB*, AB079499; *Nt\_rbohD*, AF506374; *Nt\_rbohF*, AJ302505; *St\_rbohA*, AB050600; *St\_rbohB*, AB050661; *St\_rbohC*, AB198716; *St\_rbohD*, AB198717; and *St\_rbohF*, AB064343.

12 nectary tissues (Fig. 7C) or in immature nectary tissues (data not shown). To confirm the expression pattern of *NEC1*, we performed immunolocalization of the *NEC1* protein using anti-*NEC1* antiserum. Labeling was poor or nonexistent in immature Stage 6 nectaries (data not shown), but was strong in Stage 12 mature nectaries (Fig. 7D). When preimmune antiserum was substituted for the anti-*NEC1* antiserum (Fig. 7E), no labeling of the tissues was observed.

A similar in situ hybridization study was performed using a 310-nucleotide fragment of the NOX1 mRNA subcloned into a pGEM vector, pRT539. In pRT539, the SP6 promoter produced the antisense strand, while the T7 promoter produced the sense strand. As shown in Figure 8 (A and B and insets), there is positive staining





**Figure 7.** Localization of *NEC1* mRNA and protein in nectaries. A, Longitudinal paraffin section of tobacco nectary tissues at Stage 12 of development probed with antisense strand of the *NEC1* clone showing positive localization (blue coloration) of *NEC1* mRNA in special parenchyma cells. B, Higher magnification of A showing expression of *NEC1* mRNA. C, Sense control for *NEC1* mRNA. D, Resin section showing positive immunocytochemical reaction for *NEC1* protein in nectary cells. Silver-enhanced gold (bright spots in phase contrast mode) occurs in cytoplasm and is concentrated near and in cell walls. E, Adjacent control section to that in D but with primary antibody deleted from procedure. Bars = 200  $\mu\text{m}$  on A; 50  $\mu\text{m}$  on B and C; and 25  $\mu\text{m}$  on D and E.

of both nectary tissues at Stage 12 using the antisense-labeled probe. Blue staining occurs specifically localized in the cytoplasm in the form of discrete blue particles. Figure 8C serves as the sense control and is devoid of any staining. At Stage 6 (Fig. 8D) the antisense probe is localized in the inner portion of the special parenchyma tissue but not in the outer portion or the epidermis. We used the same procedure on the taxonomically unrelated floral nectary of soybean (*Glycine max*; Horner et al., 2003) at the comparable Stage 12 of tobacco (just prior to anthesis) and obtained the same blue-staining particles in the special parenchyma tissue cytoplasm (Fig. 8, E and F). Its sense control was also negative. In contrast, similar sections of soybean nectaries were tested for expression of *NEC1* mRNA and protein probes, both with negative results (data not shown). We conclude that expression of the *NOX1* mRNA occurs as early as Stage 6 in tobacco and becomes more intense by Stage 12.

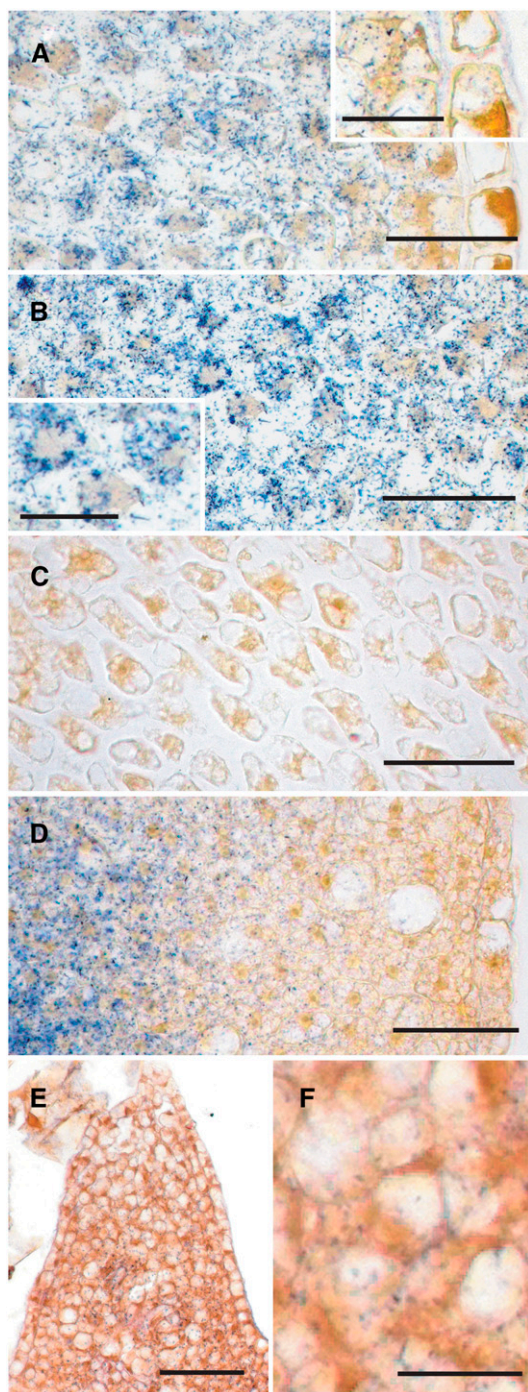
## DISCUSSION

The nectar redox cycle has been proposed to be a plant defense system that functions in ornamental tobacco to maintain nectar in a microbe-free state (Carter et al., 1999; Carter and Thornburg, 2000, 2004a, 2004b, 2004c; Thornburg et al., 2003; Naqvi et al., 2005). Here, we directly evaluated ornamental

tobacco nectar for its impact on the growth and survival of several bacterial species and found that the  $\text{H}_2\text{O}_2$  formed by the action of the nectar redox cycle can be a major inhibitor of microbial growth in nectar.

Several species of bacteria were completely inhibited by the  $\text{H}_2\text{O}_2$  in nectar. Specific destruction of the  $\text{H}_2\text{O}_2$  using catalase resulted in nectar that no longer inhibited the growth of these species. Moreover, these species, including *P. syringae*, *P. fluorescens*, and *S. typhimurium*, were sensitive to  $\text{H}_2\text{O}_2$  concentrations that have been observed in ornamental tobacco nectar (Carter and Thornburg, 2000). These findings are consistent with a role for the nectar redox cycle in microbial growth inhibition. In contrast to these species, *E. amylovora* and *P. agglomerans* (previously *E. herbicola*) grew well in untreated nectar and were resistant to  $\text{H}_2\text{O}_2$  at the concentrations found in nectar. *E. amylovora* is exceptional among bacterial plant pathogens in its ability to infect plants through the nectary stomata (Bubán et al., 2003; Mihalik et al., 2003). Although the majority of *E. amylovora* growth is believed to occur on the stigma, infection occurs only after the bacteria migrate, or are washed, into the nectar (Thomson, 1986); thus, survival in the nectar is critical to pathogenesis, and growth in the nectar is likely to be of only secondary importance. Our results suggest that tolerance to oxidative stress may contribute to the unique etiology of this pathogen, and,





**Figure 8.** In situ localization of *NOX1* in nectaries. A and B, Tobacco nectaries at Stage 12 of development probed with antisense strand of the *NOX1* clone. Inset of A shows that cytoplasmic strands are positively stained in epidermis. C, Tobacco nectaries at Stage 12 of development probed with the sense strand of the *NOX1* clone showing cells of nectary are not stained. D, Tobacco nectaries at Stage 6 of development probed with antisense strand of the *NOX1* clone. Epidermis is to the right. Staining does not extend out to the epidermis at this earlier stage. E, Soybean nectaries at an active stage probed with antisense strand of the *NOX1* clone. F, Higher magnification of the central portion of soybean nectary showing a similar cytoplasmic staining pattern as that seen in E. Bars = 50  $\mu\text{m}$  for A to E; 25  $\mu\text{m}$  for A insert and B insert; and 20  $\mu\text{m}$  for F.

furthermore, that evaluating the presence of superoxide and  $\text{H}_2\text{O}_2$  in the nectar/nectaries of fire blight-resistant and -susceptible cultivars could provide insights into traits contributing to host resistance.

Strains of two of the species tested, *P. agglomerans* and *P. fluorescens*, are commonly used as antagonists of *E. amylovora*. Successful biological control results primarily from the antagonist colonizing the stigma and preventing subsequent colonization by the pathogen (Wilson et al., 1992; Johnson and Stockwell, 1998). Therefore, growth or survival of an antagonist in the nectar is unlikely to be required for effective control. This may explain why *P. fluorescens* strain A506, which is commercially available for biological control of fire blight (BlightBan A506; NuFarm), is effective despite its poor survival in nectar. The strong growth and survival of the *P. agglomerans* in the nectar, however, may reflect an additional opportunity for competitive exclusion of the pathogen, as well as reflects the close taxonomic relatedness between *E. amylovora* and *P. agglomerans*, particularly among the species examined.

The production of  $\text{H}_2\text{O}_2$  for the nectar redox cycle has been proposed to occur via a two-step process in which molecular oxygen is first reduced to superoxide and superoxide is disproportionated to  $\text{H}_2\text{O}_2$  and molecular oxygen (Eqs. 1 and 2). Because of its importance as a substrate in the nectar redox cycle, we have sought to identify a source of the superoxide in ornamental tobacco nectaries. Associated with the nectary we identified regions opposite each other that stained intensely for superoxide. These regions, known as the nectary pore regions, are stomata rich and are the sites of nectar secretion. Our microscopy results reveal that superoxide production is limited to the vicinity of the nectary pores. As nectar is secreted through them, limitation of superoxide production to these regions protects the bulk of the nectary from possible toxic effects of superoxide and facilitates accumulation of superoxide in soluble, secreted nectar for use in the nectar redox cycle.

Superoxide is generally produced in plants by one of two different enzyme systems, NADPH oxidase or NADPH peroxidase. Both of these enzymes are inhibited by DPI; however, they can be differentiated based on the fact that NADPH peroxidase is inhibited by sodium azide and sodium cyanide, while NADPH oxidase is not (Van Gestelen et al., 1997; Bolwell et al., 1998). Because neither of these metabolic inhibitors blocked superoxide production, we concluded that the superoxide is generated by an NADPH oxidase.

The temporal expression of *NEC1*, which encodes the Nectarin I superoxide dismutase, correlated with that of superoxide accumulation and thus with NADPH oxidase activity. Nectarin I is the germin-like superoxide dismutase that disproportionates superoxide to  $\text{H}_2\text{O}_2$  and molecular oxygen (Carter et al., 1999; Carter and Thornburg, 2000, 2004a). The expression of NADPH oxidase activity is coordinated with the expression of the Nectarin I protein. However, in situ hybridization demonstrates that expression of the



nectary NADPH oxidase mRNA occurs earlier than the *NEC1* mRNA. Therefore, some form of posttranslational regulation of the NADPH oxidase likely occurs in the nectary to limit its activity. This is also not surprising since NADPH oxidase activity is generally regulated by posttranslational mechanisms. Indeed recent studies indicate that a small-molecular-mass GTP-binding protein, RAC, may function to regulate NADPH oxidase activity in both plants and animals (Heyworth et al., 1993; Kieffer et al., 1997; Park et al., 2000; Bokoch and Diebold, 2002). A recent expressed sequence tag study in ornamental tobacco nectaries has identified a homolog of the tobacco *RAC2* protein that is involved in the elicitor-induced oxidative burst (Kieffer et al., 1997). Further studies on this gene are in progress.

The ornamental tobacco nectar proteome consists of five proteins, Nectarins I to V. Nectarins I, III, and V function in the Carter-Thornburg nectar redox cycle, to produce very high levels of  $H_2O_2$ . As described above, Nectarin I is a superoxide dismutase that functions to disproportionate superoxide into  $H_2O_2$  and molecular oxygen (Carter et al., 1999; Carter and Thornburg, 2000). Nectarin II is a nonactive proteolytic cleavage product of Nectarin III. Nectarin III is a bifunctional enzyme with monodehydroascorbate reductase activity as well as carbonic anhydrase activity. We propose that the monodehydroascorbate reductase activity functions to convert monodehydroascorbate, produced from the detoxification of hydroxyl free radicals, to ascorbate in nectar. The carbonic anhydrase activity buffers the pH of nectar with bicarbonate, the same buffer found in blood (Carter and Thornburg, 2004b). Nectarin V is a Glc oxidase that also generates  $H_2O_2$  from free oxygen via the oxidation of Glc, apparently to gluconic acid. Nectarin V can also use dehydroascorbate in place of oxygen as a terminal electron acceptor, thereby converting dehydroascorbate back to ascorbate (Carter and Thornburg, 2004c). Nectarin IV does not function in the nectar redox cycle; instead, it is an antifungal defense protein that inhibits hemicellulose-degrading fungi (Naqvi et al., 2005). The inclusion of the nectary-expressed NADPH oxidase in the nectar redox cycle completes the enzymatic activities required for production of high levels of  $H_2O_2$  and for the regeneration of ascorbate to maintain the cyclical nature of the pathway. Thus, each of the proteins that accumulate in the nectar of ornamental tobacco may be considered to be a defense protein, implying that the protection of the gynoecium and the ovules/developing seeds contained therein is an extremely important function of nectar in plants.

The problem of microbial growth in nectar has been solved independently within the genus *Allium*. Two defense-related proteins were identified in the nectar of leek (*Allium porrum*) plants (Peumans et al., 1997). A Man-binding lectin that accumulated to approximately 150  $\mu\text{g}/\text{mL}$  of nectar was identified. This highly conserved protein has antibiotic properties against nematodes and insects with piercing-sucking mouth-

parts (Hilder et al., 1995; Powell et al., 1995; Rabhé et al., 1995). A second defense-related protein isolated from leek nectar is alliinase (alliin lyase), a pyridoxal phosphate-containing enzyme that produces the chemicals responsible for the pungent odor of *Allium* plants. Alliin and its related compounds are known for their antimicrobial activity against a wide variety of organisms (Gram-negative and Gram-positive bacteria, fungi, parasites, and viruses; Ankri and Mirelman, 1999). Thus, it appears that microbial growth in nectar is an important problem that has provoked at least two separate mechanisms for maintaining microbe-free nectar among the angiosperms. It will be interesting to learn in the future whether other mechanisms for nectar protection have also evolved and how widespread these or other similar mechanisms are in the flowering plants.

## MATERIALS AND METHODS

### Materials

Chemicals and reagents used in these experiments were obtained from Sigma Chemical or from Fisher Chemical. Other materials were obtained locally and were of the highest quality obtainable. The anti-NEC1 antiserum was previously described (Carter et al., 1999).

### Plants

The *Nicotiana langsdorffii*  $\times$  *Nicotiana sanderae* Hort. var Sutton's Scarlett line LxS8 ornamental tobacco plants used for the production of Nectarin I and the procedures for the isolation of nectar and floral tissues were described previously (Carter et al., 1999). If not used immediately, tissues were maintained at  $-20^\circ\text{C}$  until use. The Tr461 transgenic tobacco plants expressing the Nectarin I-chloramphenicol acetyl transferase gene were also described previously (Carter and Thornburg, 2003). CAT assays were performed exactly as described previously (Carter and Thornburg, 2003).

### Microbial Growth Inhibition

The impact of nectar on the growth of a  $H_2O_2$ -sensitive, *oxyR*-deficient *Escherichia coli* (Weinstein-Fischer et al., 2000) was evaluated on Luria-Bertani (LB) agar (Miller, 1972). This strain MC4100 *oxyR::kan E. coli* strain was kindly provided to us by Dr. Shoshy Altuvia of the Hebrew University in Jerusalem. Ten microliters of fresh nectar, which was collected as described below and was either treated with 1  $\mu\text{L}$  of 1 mg/mL catalase for 10 min or left untreated, was placed in a 0.5-cm hole punched in the agar of a plate supporting a freshly introduced bacterial lawn of cells. The plates were incubated overnight at  $37^\circ\text{C}$  and examined the following morning.

The ability of bacteria to grow directly in nectar was examined for *Pseudomonas fluorescens* strain A506 (Wilson and Lindow, 1993), *Pseudomonas syringae* strain B728a (Loper and Lindow, 1987), *Erwinia amylovora* strain Ea8R (Wilson and Lindow, 1993), *Pantoea agglomerans* strain BRT98 (Marcell and Beattie, 2002), and *Salmonella typhimurium* strain ST1R (O'Brien and Lindow, 1989). These strains were all resistant to rifampicin. Bacterial cultures were grown at  $28^\circ\text{C}$  in LB medium containing rifampicin (25  $\mu\text{g}/\text{mL}$ ; LB<sub>Rif</sub>). Approximately  $10^6$  cells, prepared using 10 mM phosphate buffer (pH 7) as diluent, were introduced into 0.5 mL of prepared nectar. Nectar was collected from mature greenhouse-grown ornamental tobacco flowers. After removing the flower and receptacle from the peduncle, the nectar was squeezed from the remaining receptacle into a collection vial. The nectar was filter-sterilized and stored at  $-20^\circ\text{C}$  for up to 10 d. Five-milliliter aliquots of thawed nectar were amended with 250  $\mu\text{L}$  of catalase (final concentration 0.5 mg/mL) or sterile water and were placed on a shaker for 20 min. Bacteria were introduced into triplicate samples of nectar, the cultures were incubated at  $28^\circ\text{C}$  with shaking, and the bacteria in 20- $\mu\text{L}$  samples were enumerated by dilution plating on LB<sub>Rif</sub> agar.

Bacterial tolerance to H<sub>2</sub>O<sub>2</sub> was evaluated by transferring cells that were grown in LB<sub>Rif</sub> medium to MinA medium (Miller, 1972) containing 0.2% Suc as the sole carbon source and 0, 4, or 40 mM H<sub>2</sub>O<sub>2</sub>. Cells were enumerated on LB<sub>Rif</sub> agar prior to and immediately following transfer, and at 4 and 24 h after transfer.

## Cloning of a NADPH Oxidase Subunit Fragment

RNA was isolated from Stage 12 nectaries (flowers at anthesis) by the method of Chomczynski and Sacchi (1987), reverse transcribed according to standard methods using oligo(dT) (Ausubel et al., 1992), and the first-strand cDNA was used for RT-PCR using one pair of degenerate oligonucleotides, nox1 and nox8 (5'-ACNCGNTTYAAYGCNTTYTGGTA-3' and 5'-NGGN-GTNGCNCCDATNCC-3'), where N = G + A + T + C, Y = T + C, and D = A + G + T. The resulting major fragment was cloned into the *Hinc*II site of pUC9 and sequenced at the Iowa State University Nucleic Acid Facility. The sequence of the LxS8 *NOX1* clone was deposited in GenBank with accession number DQ497543.

## Native PAGE Analysis of NADPH Oxidase

NADPH oxidase activity was analyzed using a PAGE system that was specific for NADPH-dependent superoxide generation (Sagi and Fluhr, 2001). For these assays, nectaries were isolated as described (Carter et al., 1999) and immediately (within 15 s of harvest) homogenized in 50 mM sodium phosphate buffer, pH 6.8, and containing 0.5% Triton X-100. This high level of triton aids in solubilizing membrane proteins, including NADPH-dependent redox enzymes (Serrano et al., 1994). By harvesting these tissues on ice and immediately homogenizing them, we have been able to avoid the wound-inducible activation of NADPH oxidase (Keller et al., 1998; Sagi and Fluhr, 2001). An aliquot containing 100 µg of protein from each tissue homogenate was electrophoresed on a 10% native polyacrylamide gel. After electrophoresis, the gels are subjected to an in-gel NADPH-dependent oxidase staining procedure using NBT to detect superoxide production (Frahry and Schopfer, 2001). The in-gel staining solution contains 0.5 mg/mL NBT in 10 mM Tris, pH 7.4, and 134 µM NADPH.

## NBT Staining of Nectary Tissues

Staining of the ornamental tobacco nectaries with NBT was performed using flowers still attached to plants. The flowers were tied in a vertical position and about 500 µL of 0.5 mg/mL NBT in 10 mM Tris-HCl, pH 7.4, was added into the floral tube. After several hours to overnight, the flowers were harvested, and the nectaries were dissected and photographed. For the inhibition studies, 50 µM DPI was included in the NBT solution and flowers were processed exactly as described.

## In Situ Hybridization

To prepare the probes for in situ hybridization, small gene fragments of approximately 320 nucleotides were amplified from the cDNAs using gene-specific oligonucleotides (*NEC1*-1, 5'-TGTACTGGTCTCTAAGAAAA-3' and *NEC1*-2, 5'-GTAAACCCCTCTCAATTGAAA-3'; *NOX1*-F, 5'-TAACGCTTT-TTGGTACTCTCA-3' and *NOX1*-R, 5'-GCCACTCAAATGGAGAAACT-3'). The resulting PCR products were subcloned into transcription vectors. For *NEC1*, a 318-nucleotide fragment (from nucleotides 494–811 of the cDNA; 37% GC content) was cloned into pGEM-T to make the vector pRT538. The Sp6 promoter produced the sense strand that should not hybridize to the mRNA and the T7 promoter produced the antisense strand that should hybridize to the mRNA. For *NOX1*, a 310-nucleotide fragment (corresponding to nucleotides 1,648–1,957 of *Nicotiana tabacum* NADPH oxidase *rbodD* AF506374; 40.1% GC content) was cloned into pGEM-T to make the vector pRT539, such that the Sp6 promoter produced the antisense strand that should hybridize to the mRNA and the T7 promoter produced the sense strand that should not hybridize to the mRNA.

Tobacco nectaries from flowers at Stage 6 and Stage 12 (Koltunow et al., 1990) and soy nectaries from *Glycine gracilis* at or just prior to anthesis were fixed on ice under vacuum in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 6.8, and embedded in paraffin. Sections cut 10-µm thick were mounted on ProbeOn Plus slides (www.fishersci.com), deparaffinized, rehydrated, and prepared for hybridization following the

protocol of Leitch et al. (1994). The Sp6 antisense and T7 sense strands were hybridized to the appropriate sections. Color detection was achieved with a DIG Nucleic Acid Detection kit from Roche Diagnostics (formerly Boehringer Mannheim), with the following modifications to the manufacturer's protocol: 1% (w/v) bovine serum albumin and 0.3% (v/v) Triton X-100 were added to blocking buffer 2, and 5 mM levamisole was added to the alkaline phosphatase substrate solution to prevent binding to any indigenous alkaline phosphatase in the sections. Controls included sections exposed to the sense probe, sections exposed to the antibody but to neither probe, and sections exposed to either probe but not to the antibody. Probes were labeled with DIG using Roche DIG RNA Labeling kit (catalog no. 1 175 025).

## Immunocytochemistry

Tobacco S6 and S12 nectaries and soy nectaries at anthesis were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde, infiltrated and embedded with LR White Resin (London Resin) as recommended by the manufacturer, and 1-µm-thick sections were adhered to ProbeOn Plus slides (Fisher Chemical). Following standard immunocytochemistry procedures, sections were exposed to 1:500 anti-LxS8\_Nectarin 1 overnight, followed by 1:100 gold-labeled secondary antibody for 2 h, followed by silver enhancement (Goldmark Biologicals; <http://users.aol.com/goldmarker/>). Control sections that were not exposed to the primary antibody were otherwise treated the same.

## Microscopy and Imaging

Bright-field and phase contrast images were digitally captured with a Zeiss Axiocam MRC camera (www.zeiss.com) mounted on an Olympus B40 compound microscope (www.olympusamerica.com) using Zeiss Axiovision AC 4.2 software. Images were processed in Adobe PhotoShop and grouped and labeled in either Adobe Illustrator or Macromedia Freehand.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number DQ497543.

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